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lola Has the Properties of a Master Regulator of Axon–Target Interaction for SNb Motor Axons of *Drosophila*

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The proper pathfinding and target recognition of an axon requires the precisely choreographed expression of a multitude of guidance factors: instructive and permissive, positive and negative, and secreted and membrane bound. We show here that the transcription factor LOLA is required for pathfinding and targeting of the SNb motor nerve in *Drosophila*. We also show that *lola* is a dose-dependent regulator of SNb development: by varying the expression of one *lola* isoform we can progressively titrate the extent of interaction of SNb motor axons with their target muscles, from no interaction at all, through wild-type patterning, to apparent hyperinnervation. The phenotypes we observe from altered expression of LOLA suggest that this protein may help orchestrate the coordinated expression of the genes required for faithful SNb development. © 1999 Academic Press

Key Words: **axon guidance; synapse specification; alternative splicing; transcription factor; cell adhesion.**

INTRODUCTION

As an axon projects toward its synaptic targets *in vivo*, it encounters a series of guidance "choice points," at each of which the axon can either continue growing, turn onto a different substratum, or stop and form a synapse. It is generally believed that at each of these choice points the axonal growth cone integrates signals from a large number of guidance cues, including both attractive and repulsive factors, both diffusible and membrane-bound (Bixby *et al.*, 1987; Dodd and Jessell, 1988; Tessier-Lavigne and Goodman, 1996; Winberg *et al.*, 1998a). This view of axon guidance, however, introduces its own complexity: what mechanism orchestrates the expression of the very precise constellation of guidance cues, receptors, and signaling proteins required to specify a particular guidance choice (Daston and Koester, 1996)? The magnitude of the problem is best illustrated by considering a particularly well-studied example of axon targeting, the pathfinding and target recognition performed by axons of the SNb motor nerve of the *Drosophila* embryo.

In each embryonic abdominal hemisegment, the motor fascicle of SNb (also known as ISNb) comprises the axons of eight identified motoneurons that project dorsally out of the ventral nerve cord, along a reproducible trajectory, to yield a precise pattern of innervation of seven bodywall muscles: the four ventral longitudinal muscles and the three most dorsal of the ventral oblique muscles (Sink and Whitington, 1991; Landgraf *et al.*, 1997; diagrammed schematically in Fig. 1H). Genetic studies have to date identified at least 13 different cell surface and secreted proteins whose pattern and level of expression contribute to the faithful pathfinding of SNb motor axons and to the selection of their appropriate synaptic targets (Van Vactor *et al.*, 1993; Lin and Goodman, 1994; Nose *et al.*, 1994; Chiba *et al.*, 1995; Matthes *et al.*, 1995; Fambrough and Goodman, 1996; Desai *et al.*, 1997; Rose *et al.*, 1997; Shishido *et al.*, 1998; Winberg *et al.*, 1998a, b; Yu *et al.*, 1998). Molecular data potentially implicate a number of additional cell surface proteins in these processes, as well.

Several proteins have been shown to play precise, instructive roles in defining the synaptic choices of particular SNb motoneurons. For example, recognition of muscles 6 and 7 by the RP3 motoneuron derives in part from expression of the chemoattractant Netrin B by these two muscles and presumably also from expression of the Netrin receptor in

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the RP3 growth cone (Mitchell *et al.,* 1996; Winberg *et al.,* 1998a). Additional specificity of RP3 targetting is provided by the homophilic interactions of Fasciclin 3 protein, which is present both on the RP3 growth cone and on its two target muscles, but not on neighboring muscles (Chiba *et al.,* 1995). Similarly, the protein Capricious is expressed both in muscle 12 and in its innervating motoneuron RP5, and this complementary pattern of expression is required for faithful selection of muscle 12 by the RP5 growth cone (Shishido *et al.,* 1998).

In addition to these very specific, localized proteins, there is a substantial list of cell surface proteins that are more widely expressed and seem to play essentially permissive roles in SNb targetting. For example, the Fasciclin 2 protein is expressed on all motor axons and seems to contribute to axon–axon adhesion (Lin and Goodman, 1994). Overexpressing Fasciclin 2 in SNb motoneurons prevents SNb axons from entering the ventral muscle field or from properly interacting with their intermediate and final targets, apparently due to excessively high levels of Fasciclin 2-mediated axon–axon adhesion. The deleterious effect of increased Fasciclin 2 can be counteracted, however, by increased expression of any of a variety of anti-adhesive axonal proteins, such as the secreted protein Beat (Fambrough and Goodman, 1996; Winberg *et al.,* 1998a). Conversely, if axon–axon adhesion is reduced too much, then individual axons interact inappropriately with muscles they would normally ignore (Van Vactor *et al.,* 1993; Fambrough and Goodman, 1996; Winberg *et al.,* 1998a; Yu *et al.,* 1998). Thus, it appears that specificity of innervation is achieved, in part, by establishing a threshold of interaction which approaching axons must attain with their prospective targets in order to overcome a variety of nonspecific negative influences (Matthes *et al.,* 1995; Desai *et al.,* 1997).

It appears that reliable neuromuscular recognition requires a delicate balance of competing positive and negative factors (Fambrough and Goodman, 1996; Desai *et al.,* 1997; Winberg *et al.,* 1998a) and that these must be displayed in a precise temporal (Rose *et al.,* 1997) and spatial arrangement. Increase or decrease of any single one of either the general or the interaction-specific proteins, either attractive or repulsive, compromises the specificity of axon targetting. This implies, however, that the outcome of each guidance decision is defined by a precise pattern of expression of a particular combination of guidance genes. It therefore seems inescapable that there must be nuclear regulatory proteins whose function it is to orchestrate the appropriate expression of the set of guiding factors, receptors, and signaling proteins that together execute the guidance decisions of a given growth cone.

There are two general classes of models that can account for the coordinated expression of proteins that specify a guidance decision. It may be that each guidance protein is regulated independently, such that the overall environment at an axonal choice point emerges from the summed effects of many unrelated transcriptional programs. Alternatively, it may be that for some guidance decisions there exist a small number of "master" regulatory genes, transcription factors that directly and coordinately control the expression of multiple guidance genes and thereby ensure that they are coexpressed at the proper time, place, and relative level.

The gene *lola* is required for two axon patterning decisions in the developing fly embryo (Seeger *et al.,* 1993; Giniger *et al.,* 1994). In *lola* mutants, longitudinal axons of the central nervous system (CNS) fail to grow between neuromeres on a substratum of interface glia, and axons of the intersegmental nerve (ISN) of the peripheral nervous system fail to grow along the lateral peritracheal cells. In both contexts, the identities and differentiation of the affected neurons, and of their substratum cells, do not appear to be perturbed by the *lola* mutation, suggesting that *lola* is required specifically for the axon/substratum interaction. The *lola* transcription unit encodes a family of products by use of two promoters and alternative splicing (Giniger *et al.,* 1994). Three major *lola* transcripts were identified by Northern analysis of embryonic RNA, and two of these, *lola 3.8* and *lola 4.7,* were expressed at times that were potentially consistent with a role in the development of *lola*-dependent axons. *lola 4.7* is preferentially expressed in the mesoderm, including the substrata and targets of peripheral axons and is also expressed at somewhat lower levels in ectodermal derivatives. *lola 3.8* is preferentially expressed in the ectoderm, particularly in neurons.

The LOLA proteins are transcription factors (Giniger *et al.,* 1994; Cavarec *et al.,* 1997). Both *lola* RNAs encode proteins bearing at their amino terminus a common dimerization domain, known variously as a BTB or POZ domain. *In vitro,* BTB domains can mediate both homo- and heterodimerization (Bardwell and Treisman, 1994), and the crystal structure of the BTB domain from the human protein PLZF reveals the domain to form a tightly interwound structure which is only stable as an oligomer (Ahmad *et al.,* 1998). Like many BTB-containing proteins, *lola 4.7* also encodes a C-terminal extension bearing two zinc fingers, suggesting that the protein may bind DNA (Giniger *et al.,* 1994). This suggestion was confirmed when a protein was isolated from *Drosophila hydei* based on its binding and transcriptional activation of an enhancer in the 5′ UTR of the *copia* retrotransposon and was found to be the *lola* homolog in *D. hydei* (Cavarec *et al.,* 1997). Subsequent analysis indeed showed *copia* expression to be strongly affected in *lola* mutant embryos of *D. melanogaster.* Interestingly, however, whereas *copia* expression is decreased in the gonads of *lola* mutant embryos, suggestive of transcriptional activation by LOLA, *copia* expression is increased in mutant CNS, suggestive of transcriptional repression by LOLA (Cavarec *et al.,* 1997). Perhaps LOLA recruits cell-type-specific factors which modify its effects on transcription, much as some mammalian BTB domains recruit transcriptional corepressors, including SMRT and N-CoR (Hong *et al.,* 1997; Huynh and Bardwell, 1998). In contrast to *lola 4.7, lola 3.8* encodes no discernable DNA-binding motif. Both LOLA isoforms are found in the nucleus.

We now show that *lola* is required for pathfinding and target recognition of SNb motor axons in the developing fly embryo. We then investigate the effect of changing the level of *lola* expression in neurons or in target cells and we find that by varying *lola* expression we can, in a dose-dependent way, titrate the extent of interaction of SNb axons with the muscles of their target field, from no innervation, through wild-type patterning, and finally to apparent hyperinnervation. We suggest that this is the behavior one might predict for a "master" regulator of SNb neuromuscular interactions.

MATERIALS AND METHODS

Drosophila stocks. *lola* alleles *5D2* and *1A4* are insertions of the P-element PlacW and were described by Giniger *et al.* (1994). *lola* alleles *c46*, *e76* and *g9* were chemically induced and were generously provided by Tom Schwarz and Mike Forte; analysis of the mutant phenotypes and residual LOLA expression in these three alleles demonstrated that they are strong mutations and that they produce all the characteristic *lola* axonal phenotypes (CNS longitudinal interruptions, ISN stalling, and failure of SNb innervation; data not shown). *GAL4* lines used for expression studies were *twist-GAL4* and *GAL4-24b* (panmesodermal) and *elav-GAL4/Cyo* P[w^+; *actin-lacZ*] (all differentiated neurons). *lola* overexpression phenotypes were analyzed in F1 crosses of *UAS-lola* lines to *GAL4*-expressing lines; where necessary, embryos were double-stained with anti-β-galactosidase antibodies to identify *lola*-expressing embryos.

Construction and transformation of UAS-lola transgenes. The coding sequence of the protein isoform encoded by the *lola 4.7* splice variant was subcloned into *pUAS-T* (Brand and Perrimon, 1993) as a 3.9-kb *Eco*RI fragment from *lola* cDNA 4.8; the *lola 3.8* splice variant was subcloned as a 2-kb *Eco*RI fragment from cDNA 8.13 (Giniger *et al.*, 1994). DNA was prepared and transformed into w^{1118} flies by standard methods, and multiple independent insertions were isolated and balanced. Stocks were prepared that were homozygous for either one or two copies of each *UAS-lola* transgene. Western analysis with anti-LOLA antibodies verified that two-copy lines expressed more protein than one-copy lines when crossed to *GAL4*-producing flies (data not shown). *lola* overexpression phenotypes were verified with multiple independent insertions of the *UAS-lola* transposon.

Antibody staining and analysis. Embryos were collected and aged on grape juice agar plates at 25°C. Collections of embryos at 12.5–14.5 or 13–15 h were fixed and stained with antibodies by standard methods, with detection by peroxidase histochemistry (Vectastain Elite ABC, Vector Labs). The only exception was the Fasciclin2/Synaptotagmin double-labelling experiment, for which fluorescent detection was used. Antibodies were as follows: anti-Even-skipped (1:2), anti-Fasciclin 2 (1:375), and anti-Fasciclin 3 (1:2) (Gregg Helt and Corey Goodman); mouse anti-β-gal (1:750, Boehringer Mannheim); rabbit anti-β-gal (1:10,000, Cappel); mAb 22C10 (1:375) and anti-synaptotagmin (D-syt2; 1:1000) (Hugo Bellen); anti-muscle myosin heavy chain (1:400, Dan Kiehart); anti-S-59 (1:100, Emma Rushton and Mike Bate); and anti-Toll (1:10, Akira Chiba; and 1:500, Par Towb and Steve Wasserman). Biotinylated and fluorescently conjugated secondary antibodies were from Jackson.

Peroxidase-stained embryos were filleted with tungsten needles and mounted in 80% glycerol, or were left intact, dehydrated with ethanol, and mounted as whole mounts in JB4 embedding medium (Polysciences). Samples were examined by Nomarski microscopy and photographed with Ilford XP2 film. Negatives were scanned and the images stored on a Kodak PhotoCD. Where necessary, focal planes were montaged in Photoshop. The phenotypes reported here were observed in both filet and whole-mount preparations. Fluorescently labelled embryos were mounted in Fluorogard (BioRad) and analyzed on an MRC-600 confocal microscope (Keck Center, University of Washington).

Embryos were staged by age, by morphological criteria (particularly gut development, CNS condensation, and onset of cuticle deposition), and, where possible, by the CNS pattern of Fasciclin 2 immunoreactivity (by the criteria of Desai and Zinn; described in the Fly Motor Axon Home Page: http://www.caltech.edu/~zinn/motoraxons/fma%20home%20page.html). The CNS disruptions in *lola* mutant animals prevented us from using the Fasciclin 2 CNS pattern for staging. Therefore, samples prepared for analysis of the *lola* SNb mutant phenotype were staged using only the first two criteria, both for the mutants and for wild-type controls which were collected at the same time. anti-Fasciclin 2-stained *lola* mutant embryos were identified by their distinctive, fully penetrant CNS phenotype (Giniger *et al.*, 1994).

RESULTS

lola is required for SNb pathfinding and targeting. As described in detail by others (Johansen *et al.*, 1989; Sink and Whitington, 1991; Van Vactor *et al.*, 1993; Landgraf *et al.*, 1997), SNb motor axons initially exit the CNS through the root of the intersegmental nerve (ISN) (Fig. 1H). While the ISN projects dorsally in a relatively superficial trajectory, SNb axons separate from the ISN just ventral to muscle 28 to dive internally. They then continue projecting dorsally, between a deeper layer of ventral longitudinal muscles (VLMs) and a more superficial layer of ventral oblique muscles (VOMs), with particular axons separating from the bundle at precise points in the trajectory to innervate specific target muscles. Because of their clearer visibility in filleted embyro preparations, we have focussed our analysis on innervation of the four VLMs that are present in abdominal segments A2–A7. These are named (from ventral to dorsal) muscles 7, 6, 13, and 12. The neuromuscular junctions on the VLMs develop in the clefts between adjacent muscles during embryonic stage 17. Typically, axons infiltrate the 6/13 cleft first, followed by the 13/12 cleft, and then 7/6, although this last seems to be the most variable in timing. By the time of cuticle deposition at late stage 17, junctional material that is immunoreactive with anti-Fasciclin 2 antibody is typically visible in at least two, and often all three of the clefts (Fig. 1A, quantified in Fig. 1E).

Examination of axon patterning in *lola* mutant embryos using an antibody against Fasciclin 2 identified a previously unrecognized *lola* mutant phenotype. With complete penetrance and very high expressivity, the motor axons that project through the SNb peripheral nerve failed to form connections to their cognate muscles (Figs. 1B–1D; quantified in Figs. 1F and 1G). Most commonly, SNb axons

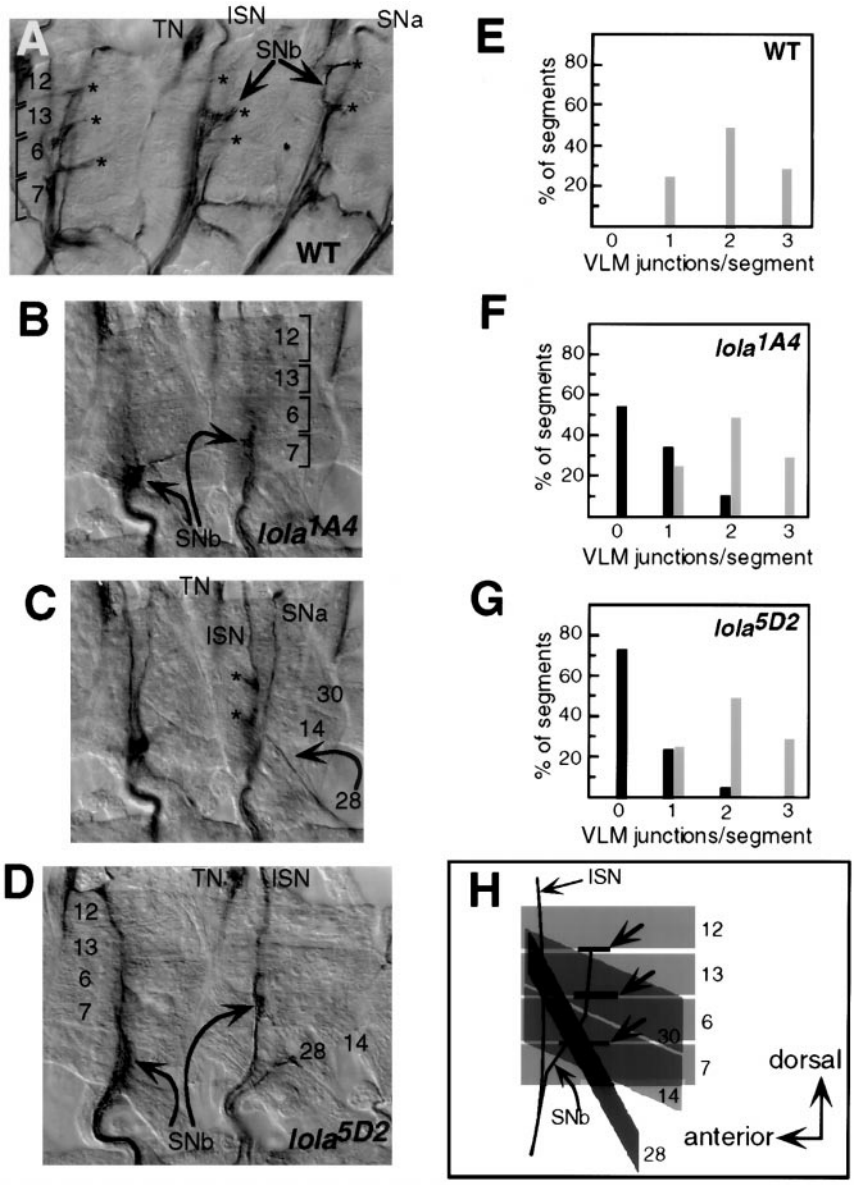

FIG. 1. *lola* is required for SNb development (A–D) SNb morphology in wild-type embryos (A, three hemisegments) or *lola* mutant embryos (B–D, two hemisegments each) was visualized by staining stage 17 embryos (13–15 h AEL) with anti-Fasciclin 2 and developing by peroxidase histochemistry. Stained embryos were filleted and mounted in glycerol; each panel shows a view of the internal surface of the ventrolateral bodywall (the SNb target field). In these and all other bodywall figures, anterior is to the left and dorsal to the top. (A) Wild-type embryo. Asterisks indicate positions of VLM neuromuscular junctions (dark, horizontal profiles in the staining). VLMs are numbered 7, 6, 13, and 12; clefts between adjacent VLMs can be discerned in the Nomarski image and are highlighted with brackets in the leftmost segment. Peripheral nerves are indicated; SNa, ISN, and TN are typically only dimly visible in the plane of focus of SNb. (B,C) Two focal planes of a $lola^{1A4}$ mutant embryo (hypomorphic allele). (B) Focused more internally to show VLM morphology. (C) Focused more superficially to show morphology of ventral oblique muscles 30, 14 and 28. SNb (arrows in B) has grown into the cleft between muscles 7 and 6 in the segment on the right, but has not grown further dorsally in either segment. The VLMs and VOMs (including the more ventral muscles 15–17, not labeled) are clearly visible in the Nomarski images. Asterisks in C indicate SNb junctions to the underlying VOMs. (D) $lola^{5D2}$ mutant embryo (strong allele). The stalled SNbs in these two segments are indicated with arrows; again, the VLMs and VOMs are visible in the Nomarski image (numbered). (E–G) Expressivity of the *lola* SNb phenotype. The number of morphologically identifiable SNb neuromuscular junctions to VLMs per hemisegment was scored and presented as a histogram for wild-type and mutant animals. (E) Development of SNb VLM junctions in nonmutant animals ($n = 65$). Homozygous balancer embryos and embryos heterozygous for the balancer over a *lola* allele were filleted and scored (gray bars). Animals scored were from the same embryo collections used for the mutant analysis of F and G. (F) SNb development in $lola^{1A4}$ embryos (black bars; $n = 29$). For comparison, wild-type values from E are shown in gray.

appeared to "stall" somewhere between the point at which they would normally separate from the ISN and the muscle 6/13 junction. In other hemisegments, SNb axons projected through the muscle field but failed to branch into the intermuscle clefts where synapses should be formed. These phenotypes were observed in embryos homozygous for either of two independent *lola* loss-of-function alleles that we examined in detail, $lola^{5D2}$ and $lola^{1A4}$. We have found previously that $lola^{1A4}$ is a hypomorphic allele, based both on the expressivity of other *lola* mutant phenotypes and on the presence of residual LOLA immunoreactivity in homozygous mutant embryos (K. Madden and E. Giniger, unpublished observations), whereas $lola^{5D2}$ is a strong allele (Giniger *et al.*, 1994). Consistent with this, we found that the effect on SNb development is slightly more severe for $lola^{5D2}$ than for $lola^{1A4}$, although most hemisegments are affected in both alleles. Both *lola* alleles decrease the expression of all *lola* isoforms and appear to have roughly equivalent effects on *lola* expression in different tissues (Giniger *et al.*, 1994; and data not shown). It seems plausible that the residual SNb development that does occur in homozygous mutant $lola^{5D2}$ individuals is due to perdurance of maternally provided protein. We attempted to test this possibility by generation of homozygous mutant germline clones (using the "dominant female sterile" method), but failed to recover any embryos from such an experiment (E. Giniger, E. Grell, S. Younger-Shepherd, and Y. N. Jan, unpublished). It may be that *lola* performs some essential function in the germline during oogenesis.

It does not appear that the *lola* SNb phenotype can be explained as resulting from a failure of specification or overall differentiation of the motoneurons or the muscles. Previous experiments from us and others have documented the wild-type expression of molecular markers for many CNS and PNS cell identities in *lola* mutant embryos (Seeger *et al.*, 1993; Giniger *et al.*, 1994). More specifically, the RP3 motoneurons that contribute to SNb can readily be identified in $lola^{1A4}$ mutant embryos stained with mAb22C10, based on their position, morphology, and initial axon trajectory (Fig. 2A). We confirmed the molecular identities of these cells as RP neurons by verifying that they express Fasciclin 3 protein (Fig. 2B). Similarly, the VUM motoneurons are also readily identified in *lola* mutants (not shown).

Since the effects of *lola* on muscle identity have not previously been analyzed in detail, we characterized muscle patterning in *lola* mutants. Perhaps the most stringent and general assay for muscle identity is the precisely reproducible pattern of muscle morphologies and attachments in the developing embryo (Bate, 1990), and for the most part these appear to be wild type in *lola* mutants. To further authenticate muscle identities in *lola* mutant embryos, we surveyed the expression of molecular markers ("founder" and "progenitor" markers; Fuerstenberg and Giniger, 1998) for several well-characterized dorsal, lateral, and ventral muscles. Figure 2C documents the expression of Even-skipped protein in myoblasts of a stage 13 *lola* mutant embryo in the position of the developing dorsal muscle 1 (Landgraf *et al.*, 1999); the inset shows Eve expression persisting in the corresponding syncitial myotube in a slightly older embryo. Similarly, in Fig. 2D, expression of the homeodomain protein S-59 is seen in a stage 13 *lola* mutant in the wild-type pattern of four clusters, reflecting its expression in the developing lateral muscles 5 and 18 (arising from clusters Ia and III, respectively), ventral muscle 25 (cluster Ib), and ventral muscles 26, 27, and 29 (cluster II) (Carmena *et al.*, 1995). Based on these data, we infer that muscle identities are not specified by *lola*. We note, however, that *lola* mutant embryos do sometimes lack one or more of the VLMs. Such defects are substantially less penetrant than is the effect of the mutation on SNb targeting, but, nonetheless, in quantifying the SNb phenotypes of $lola^{-}$ embryos we therefore restricted our analysis to hemisegments in which all four VLMs were present and had a wild-type morphology (~75–85% of hemisegments).

Finally, it seems unlikely that SNb motor axons simply fail to reach their peripheral target field as a secondary consequence of stalling earlier in their trajectory, at *lola*-dependent axonal choice points within the CNS. First, as described above, the SNb axons of *lola* mutant animals typically do reach the target field, and stall only then, when they are already in the ventral muscle domain or at its edge. Second, most of the axons which contribute to SNb do not course through CNS longitudinal nerve segments whose development depends on *lola* function (Seeger *et al.*, 1993; Giniger *et al.*, 1994; Landgraf *et al.*, 1997). We note that VUM-v contributes one axon to SNb (Landgraf *et al.*, 1997), and we have reported previously that in a modest fraction of

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Note the clear shift to fewer neuromuscular junctions per hemisegment. (G) SNb development in $lola^{5D2}$ embryos ($n = 31$). (H) A schematic depiction of the SNb target field in embryonic hemisegments A2–A7. The most internal muscles (VLMs) are depicted in the lightest gray; the most superficial muscle (VOM 28) is in the darkest gray. SNb neuromuscular junctions to the VLMs are depicted as horizontal black bars in the clefts between adjacent VLMs. For clarity, junctions to the VOMs are not shown in the schematic, though they are visible in many of the figures. Muscle 6 is depicted as if it were lying strictly adjacent to muscle 13; in the embryo it actually lies slightly internal to 13, such that the dorsal edge of 6 often extends slightly dorsal to the ventral edge of 13. Consequently, in some micrographs a faint Nomarski edge will appear parallel but slightly displaced from the indicated position of the cleft (which will highlight the edge of 13, where the only synapse is formed in this cleft). ISN is indicated for comparison; SNa and TN are not shown. In addition to the SNb phenotype, we also observed gross defects in the SNa motor nerve in *lola* mutant embryos. However, in many cases we were unable even to detect this nerve exiting the CNS, suggestive of early defects in its trajectory, so we have not characterized this phenotype further.

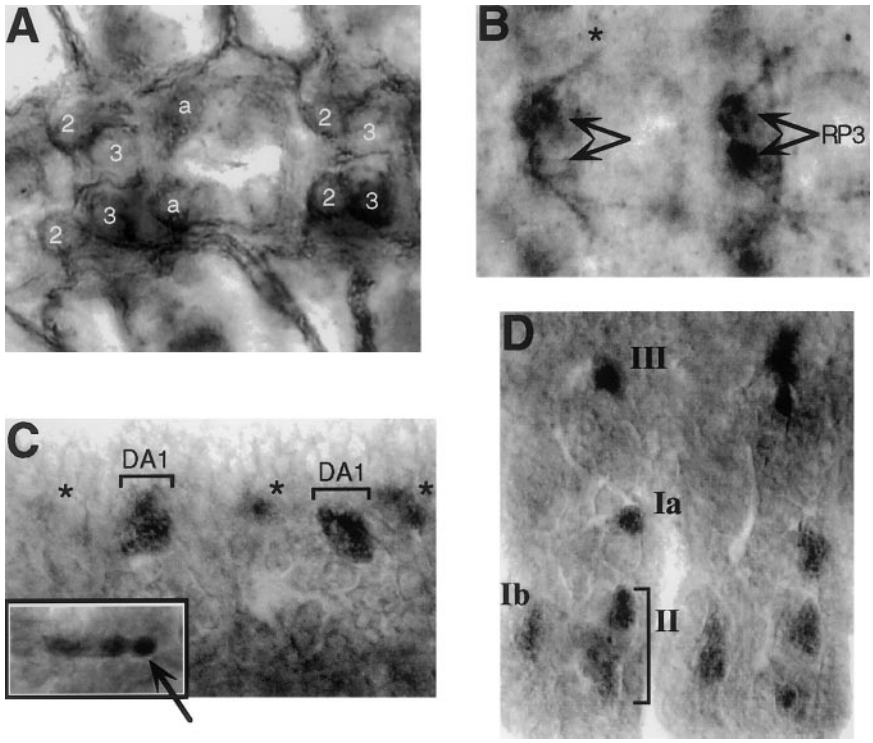

FIG. 2. Neuronal and muscle cell identities are not altered by *lola.* Embryos were incubated with the indicated antibodies and developed by peroxidase histochemistry. (A) A $lola^{1A4}$ embryo; (B–D) $lola^{c46/e76}$ embryos. (A) Dorsal view of a filleted stage 16 $lola^{1A4}$ CNS, stained with mAb 22C10. RP3 is clearly visible in all four hemisegments (denoted as "3"). The prominent motoneurons aCC ("a") and RP2 ("2") are indicated as anatomical landmarks. (B) A filleted stage 16 *lola* CNS, stained with anti-Fasciclin 3. Arrows indicate four labeled RP3 cell bodies in two segments; asterisk highlights one RP axon bundle. (C) Lateral view showing two segments of a stage 13 *lola* embryo, stained with anti-Eve. A cluster of immunoreactive myoblasts is clearly detected in the position of the developing muscle 1 (bracketed and labelled DA1 in the figure). Asterisks indicate nearby Eve-positive pericardial cells (in a slightly different focal plane). (Inset) Eve expression in a syncytial DA1 myotube after myoblast fusion (from an older embryo). (D) Lateral view of two segments from a stage 13 *lola* embryo, stained with anti-S-59. Immunoreactivity is clearly detected in the four clusters typical of the wild-type S-59 expression pattern (Ia, Ib, II, and III). At this stage, cluster II has begun to split into a dorsal portion, which will continue expressing S-59 and develop into muscle 27, and a ventral portion, which will extinguish S-59 expression and develop into muscles 26 and 29 (and perhaps also an adult muscle precursor).

hemisegments in *lola* mutants, the VUM axons fail to provide a distinct posterior root to the ISN (Giniger *et al.*, 1994). Careful examination of filleted preparations suggests that even in many of these affected hemisegments the VUM axons do reach the ISN nerve root (albeit via an abnormal trajectory; data not shown). Moreover, the SNb branch of VUM-v does not form synapses on the VLMs, but rather only on the ventral oblique muscles (Landgraf *et al.*, 1997).

Overexpression of lola in muscles causes "hyperinnervation." We reasoned that if *lola* controls the expression of one or more guidance genes, then we might gain insight into the targets, function, and site of action of *lola* by overproducing the protein in various tissues in a wild-type genetic background and assaying its effects on SNb development. Moreover, by performing this experiment with both *lola 3.8* and *lola 4.7* we hoped to begin distinguishing between the functions of these two *lola* isoforms. We therefore used the $GAL4/UAS_G$ expression system (Fischer *et al.*, 1988) to increase the levels of either *lola 3.8* or *lola 4.7*, separately, either in muscles or in differentiated neurons. We have so far detected no changes in *Drosophila* development from altered expression of *lola 3.8*, and we will not consider that isoform further in this report. In contrast, altered expression of *lola 4.7* was lethal and caused striking defects in SNb development. Overexpression of *lola 4.7* in all muscles was obtained by crossing flies that were homozygous for either one or two copies of a *UAS-lola 4.7* transgene to flies bearing the *GAL4* enhancer trap *24b;* the phenotype was assayed by collecting stage 17 embryos, and staining with anti-Fasciclin 2 to visualize motor axons. *GAL4-24b* has been extensively characterized by us and by others, and leads to reporter expression throughout the mesoderm, including all somatic muscles, but only in the mesoderm, starting well before the out-

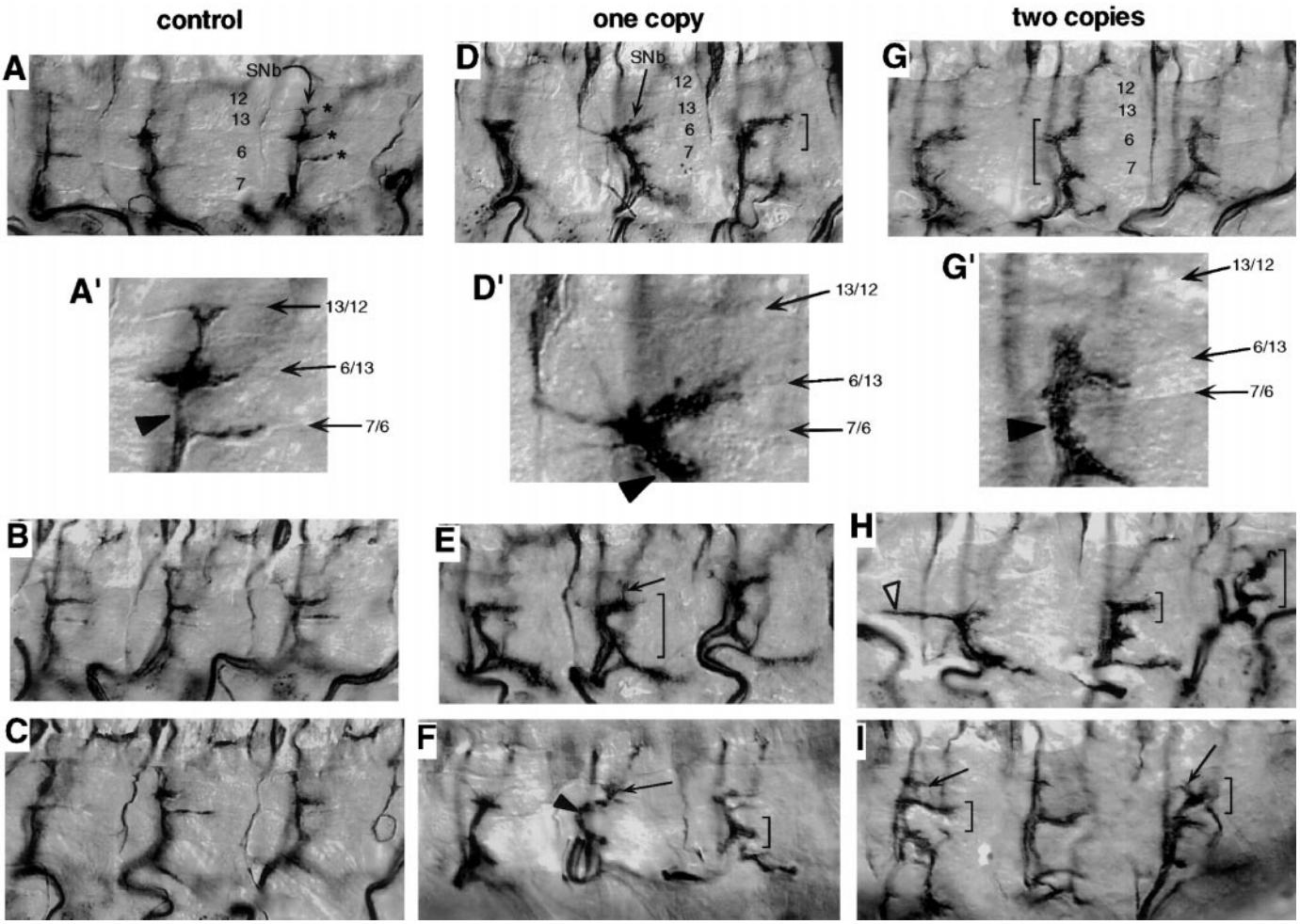

FIG. 3. Overexpression of *lola 4.7* in muscles causes "overgrowth" of SNb neuromuscular junctions. Embryos in which *lola 4.7* is or is not expressed in all muscles were prepared and analyzed by Fasciclin 2 staining. Since the phenotype from *lola* overexpression in muscles is difficult to quantify, the figure shows three hemisegments from three separate embryos for each genotype. (F, I) Whole mount, rather than filleted, embryos, documenting that muscle morphology is not perturbed by the dissection protocol. (A–C) *GAL4-24b*/+ ("control") embryos. Note the narrow junctional profiles, largely confined to the clefts between muscles (asterisks in A). Only the junction in the 6/13 cleft extends appreciably outside the cleft, and this effect is largely restricted to the immediate vicinity of the main shaft of the nerve. (A′) A higher magnification view of one hemisegment from A. Note the thin SNb nerve shaft extending from one cleft to the next (filled arrowhead). (D–F) *GAL4-24b/UAS$_G$-lola 4.7* (one copy of responder transgene). Note the thickened profiles of both the main shaft of SNb (filled arrowhead) and of the junctional material and the expansion of junctional material beyond the clefts between muscles (highlighted by a bracket in some segments). Typically, junctional material is *also* within the cleft, suggesting that the failure of proper junction morphogenesis is not likely to be secondary simply to absence of the cleft due to excessive muscle–muscle adhesion. Moreover, arrows in E and F indicate places where SNb has successfully navigated between muscles 6 and 13 to extend dorsally, again suggesting that the improper morphogenesis in these hemisgments does not reflect a simple physical barrier preventing the axon from insinuating itself between these muscles. (D′) A higher magnification view of one hemisegment from D. Clefts between successive muscles are visible in the Nomarski image. (G–I). *GAL4-24b/2x(UAS$_G$-lola 4.7)* (i.e., two copies of responder transgene). Similar phenotypes are observed as in D–F, but in a larger fraction of embryos. Again, brackets highlight regions of expanded junctional material and arrows indicate axons navigating beyond the 6/13 cleft. We occasionally observe SNb stalling at the 7/6 junction, as in H (open arrowhead). In this particular instance the neuromuscular junction has also extended anteriorly beyond the edge of the segment to continue in the 7/6 cleft in the next more anterior hemisegment.

growth of motor axons (Brand and Perrimon, 1993; Fuerstenberg and Giniger, 1998).

In embryos that overexpressed *lola 4.7* in muscles, we observed an apparent "overgrowth" of Fasciclin 2-immunoreactive material where SNb axons contacted the VLMs (Fig. 3). Some of this material was in the clefts between adjacent muscles, but more spread out of the clefts and over the surfaces of the muscles. Moreover, in many

cases, broad branches extended over the muscles quite separate from the normal projections in the clefts. Precise quantification of the phenotype is difficult because the classification of individual junctions as either wild type or abnormal is sometimes ambiguous; however, clearly aberrant phenotypes like those in the figure were observed in approximately 45% of GAL4-expressing embryos bearing one copy of the *UAS-lola* transgene and in ~66% of embryos bearing two copies of the responder transgene (n = 24 embryos of each genotype in the experiment shown). Qualitatively similar results were obtained with several independent insertions of the *UAS-lola* transgene and also with a second mesoderm-specific *GAL4* driver (*twist-GAL4*). In addition, at the highest level of expression (with two copies of the *UAS-lola* transgene) we observed very rare cases where SNb seemed to stall in the ventral part of the target field in association with the 6/7 cleft, but where no axons continued more dorsally to muscles 13 or 12 (Fig. 3H).

In some studies of SNb development that have employed overexpression of transgenes in developing muscles, axonal defects have been interpreted as arising secondary to defects in muscle morphogenesis. In particular, it has been suggested that excessive adhesion between adjacent muscles can physically block the infiltration of SNb axons between muscles, leading to axon misroutings and misplaced terminal arborizations (Nose *et al.*, 1997; Raghavan and White, 1997). It seems unlikely that such effects underly most of the phenotypes we observe. First, we do not observe junctional immunoreactivity to be excluded from the clefts between adjacent muscles, but rather to be expanded, with material both in and around the clefts. Morever, we commonly see an expansion of material at the 6/13 junction, and this occurs even in hemisegments where SNb axons successfully penetrate this cleft and continue to muscle 12 (Figs. 3E, 3F, and 3I, arrows). Third, a striking feature of the phenotype is expansion of the shaft of the nerve, particularly on muscles 6 and 7, where SNb should have the narrow caliber typical of a peripheral nerve (Fig. 3, black arrowheads in 3D′ and 3G′, and Fig. 4) Finally, the clefts between the four VLMs are clearly visible morphologically in *lola*-overexpressing embryos, both in fillet and in whole-mount preparations.

In order to test whether the large, Fasciclin 2-immunoreactive patches that we observed from *lola* overexpression were likely to be developing presynaptic specializations, we double-stained these preparations with anti-Fasciclin 2 and with an antibody against a synaptic vesicle component, Synaptotagmin (Fig. 4). We found that the Fasciclin 2 immunoreactivity was densely punctuated with small foci of intense anti-Synaptotagmin staining that are indicative of developing synaptic terminals, and this was observed even outside of the clefts where such structures are typically localized in wild-type animals (Littleton *et al.*, 1993). These data suggest that the expanded Fasciclin 2 immunoreactivity we observe is likely to reflect expanded domains of synaptogenesis. We have not yet tested whether these expanded domains of axon–muscle contact result in increased synaptic efficacy at the mature neuromuscular junctions.

Ectopic expression of lola 4.7 in neurons phenocopies the lola mutant SNb phenotype. We next overexpressed *lola 4.7* in differentiated neurons by crossing flies bearing one or two copies of *UAS-lola 4.7* to flies carrying *elav-GAL4,* a well-characterized transgene that leads to *GAL4* expression in all postmitotic neurons, relatively late in their differentiation (Luo *et al.,* 1994; Fambrough *et al.,* 1996; Giniger, 1998). We then assayed SNb development by staining embryos with anti-Fasciclin 2. We observed a phenotype that was strikingly reminiscent of the *lola* mutant phenotype, with SNb axons failing to innervate the clefts between adjacent VLMs and often stalling between the point of separation from the ISN and the muscle 6/13 junction (Figs. 5A–5D). This phenotype was dose-dependent, occurring at higher frequency when multiple copies of the *UAS-lola* transgene were present (quantified in Figs. 5E–5G). Finally, at the highest levels of expression, we very occasionally observed a "bypass" phenotype of SNb axons failing to separate from the ISN altogether (n = 2/79; Fig. 5D). As was true for the *lola* mutant phenotype, it is unlikely that the failure of SNb-VLM interaction in *elav-GAL4/UAS-lola* embryos is secondary to a failure of the neurons to differentiate, as we could readily identify the RP3 and VUM motoneurons in such embryos (not shown). We saw no evidence for defects in muscle morphogenesis in *elav-GAL4/UAS-lola 4.7* embryos.

DISCUSSION

During stage 17 of *Drosophila* embryogenesis, seven ventral bodywall muscles become innervated by eight axons which project through the SNb peripheral nerve. The genetic mechanisms that match each axon to its specific target in this system have been studied extensively, perhaps more so than any other system for analyzing synapse choice *in vivo.* Nearly 20 cell surface proteins have been identified that are expressed on some or all of these muscles, the incoming growth cones, or both, during the process of target recognition. For nearly two-thirds of these proteins, genetic experiments have demonstrated that the protein contributes to the specificity of target recognition *in vivo:* an increase or decrease in protein expression, in appropriate genetic backgrounds, disrupts the stereotyped matching of neuron to target. This wealth of data, however, raises a new and challenging question: what gene regulatory mechanisms maintain the precise balance of all these disparate positive and negative factors, to ensure that their effects add-up to specify the precisely reproducible pattern of SNb innervation?

The transcription factor LOLA is expressed both in the neurons that project through SNb and in the muscles that they innervate. We have found that *lola* is required for the interaction of SNb axons with the ventral muscles: in the

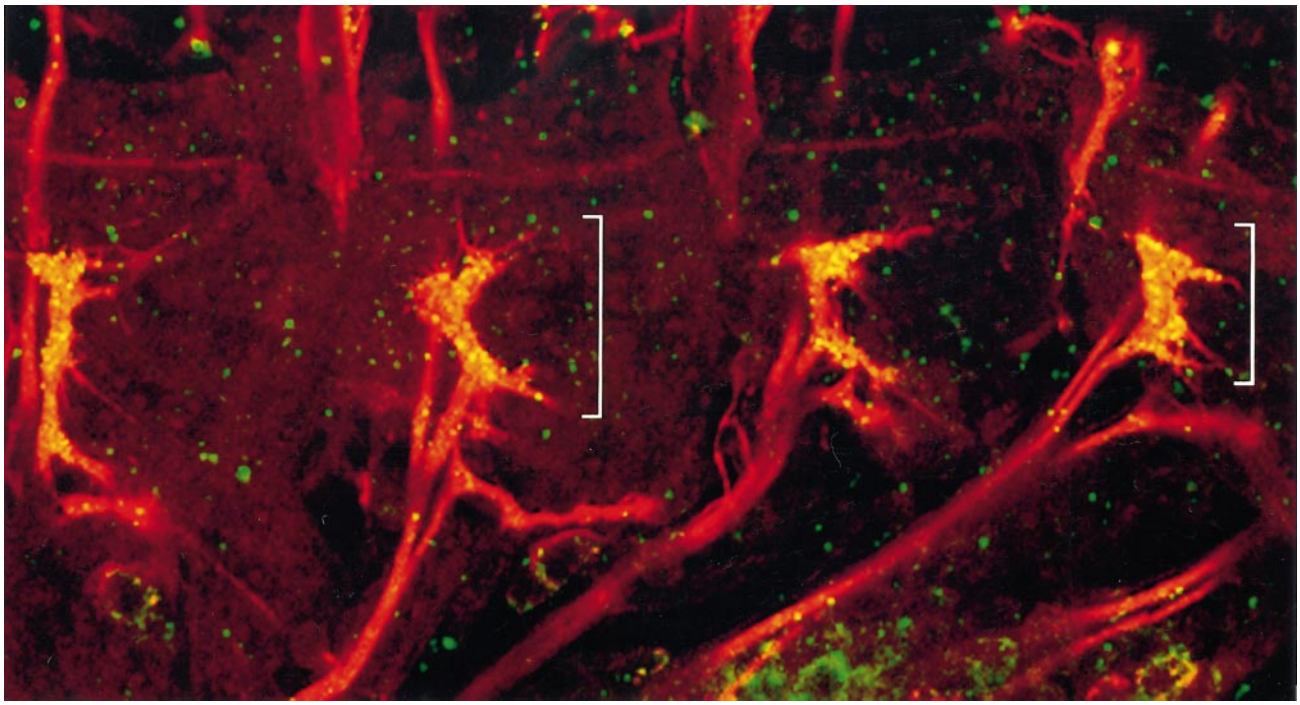

FIG. 4. Hypertrophied junctional material from *lola* overexpression contains synaptic vesicle components. *GAL4-24b/2x(UAS$_G$-lola 4.7)* embryos were collected and visualized by indirect immunofluorescence after double-staining with anti-Fasciclin 2 (Texas Red) and anti-Synaptotagmin (FITC); colocalization appears yellow. Note that punctate foci of yellow staining, reflecting concentration of Synaptotagmin at nascent active zones, are observed throughout the expanded domains of junctional material (white brackets), and not just at the positions of intermuscle clefts. In some samples, a low level of nonspecific punctate background staining is seen with this antibody in both wild-type embryos and experimental embryos (scattered green dots), but this background is easily distinguished from the concentrated signals observed in developing synaptic terminals.

absence of *lola* function, the SNb growth cones reach their muscle target field, but then stall at about the point where they should separate from the ISN and begin exploring the surfaces of the ventral muscles. Moreover, we have found that by varying the level of expression of one *lola* isoform, we can titrate the degree of interaction of SNb axons with the ventral muscles, from no interaction at all, through accurate synaptogenesis, to apparent hyperinnervation.

Given the phenotypes induced by both loss- and gain-of-function manipulations of *lola,* and the properties of LOLA protein, the simplest hypothesis is that *lola* regulates the transcription of genes that control the interaction of SNb axons with their target muscles—but which genes? It could be that *lola* regulates the expression of just one critical cell surface protein. However, no gene has been described whose phenotypes mimic both the loss- and gain-of-function effects of *lola.* For example, altered expression of *beat* can cause axon stalling at the point of SNb defasciculation, but unlike *lola* it also causes highly penetrant "bypass" phenotypes and is not reported to induce expanded terminal arbors (Fambrough and Goodman, 1996). Moreover, in a wide variety of experimental paradigms, increases in net adhesion of SNb axons arising from altered expression of single cell surface proteins result in severe axon misroutings and ectopic contacts to muscles that are not normally SNb targets (muscles 5 and 8, for example; Chiba *et al.,* 1995; Krueger *et al.,* 1996; Winberg *et al.,* 1998a). In contrast, we observe an expanded area of contact between SNb axons and their proper set of target muscles, not ectopic projection into nearby inappropriate territories, and only extremely rare axon misroutings. We note, however, that we have not yet examined at single-neuron resolution the fidelity of individual neuron–target contacts within the SNb target field in embryos that overproduce *lola* in muscles.

We speculate that a simpler explanation for the *lola* phenotypes may be that *lola* coordinately regulates the expression of a number (though not necessarily all) of the proteins that, together, control the interaction of SNb axons with the ventral muscles. Thus, in its absence neuron–muscle recognition fails altogether due to the loss of multiple axon–target interactions (Bixby *et al.,* 1987; Desai *et al.,* 1997). Conversely, altering the level of *lola* expression in a cell resets the net strength of SNb-VLM interaction by varying the *absolute* levels of a suite of cell surface proteins made by that cell, but largely maintains their *relative*

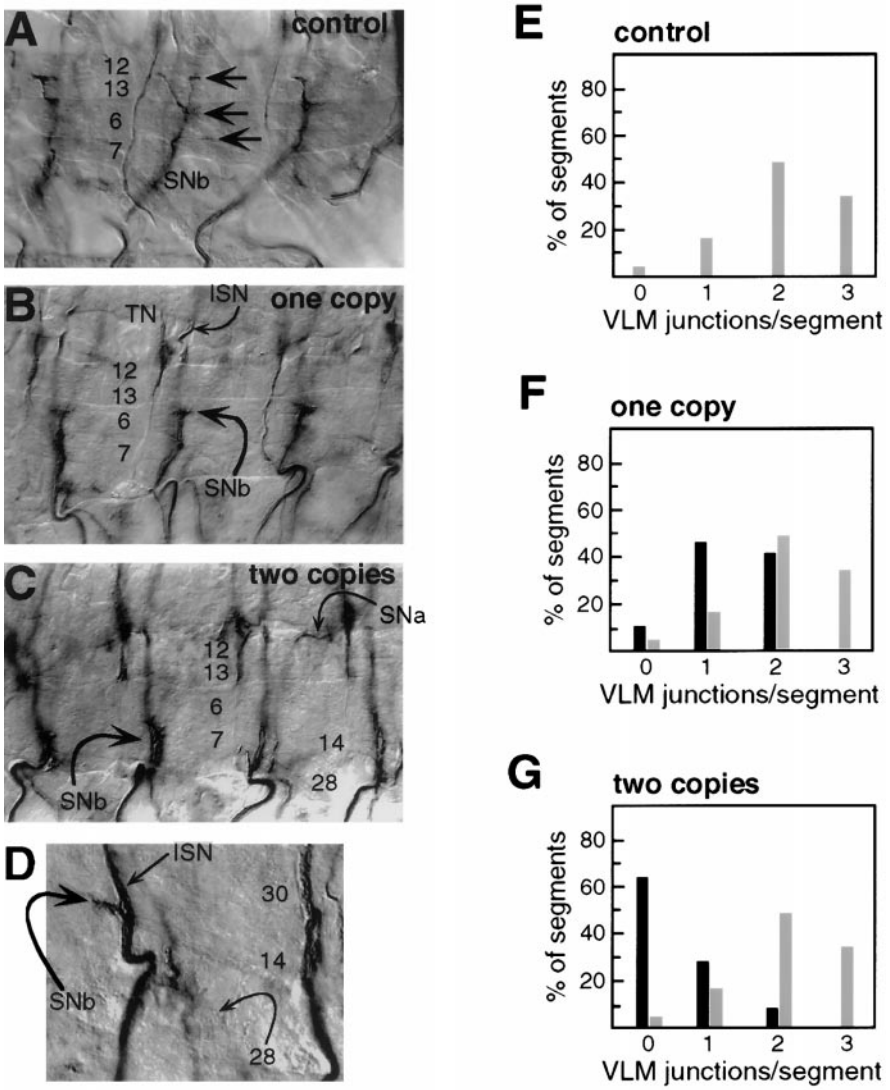

FIG. 5. Overexpression of *lola 4.7* in neurons phenocopies the *lola* mutant phenotype. Embryos in which *lola 4.7* is or is not overexpressed in all postmitotic, terminally differentiated neurons were prepared and analyzed by Fasciclin 2 staining, as described in the legend to Fig. 1. (A) *elav-GAL4/+* ("control") embryo. Arrows indicate the positions of neuromuscular junctions, and VLM Nomarski profiles are identified by number. (B) *elav-GAL4/UAS$_G$-lola 4.7* (one copy of responder transgene). Only a single SNb-VLM junction is formed in each hemisegment shown (thick arrow), other apparent junctions are to underlying VOMs. (C) *elav-GAL4/2x(UAS$_G$-lola 4.7)* (two copies of responder transgene). Complete failure of SNb-VLM junction formation; arrow indicates stalled SNb (D) A different *elav-GAL4/2x(UAS$_G$-lola 4.7)* embryo, with micrograph focused at the level of the ISN. In this embryo, SNb has formed a junction to one of the VOMs (thick arrow), but displays a "bypass" phenotype in which SNb has failed to separate from the ISN by diving under muscle 28 (thin arrow), even though that muscle and the adjacent VOM 14 are visible in the Nomarski image. (E–G) Histograms quantifying the effect of neuronal *lola 4.7* expression on development of SNb–VLM neuromuscular junctions. Analysis is as described in the legend to Fig. 1. E quantifies junction formation in *elav-GAL4/+* ($n = 110$). F quantifies *elav-GAL4* with one copy of the *UAS$_G$-lola* responder ($n = 101$) and G with two copies of the responder ($n = 79$). Control values are shown in light gray in F and G. The number of SNb-VLM junctions formed per hemisegment declines with increasing *lola* expression.

levels. By this view, the misroutings and ectopic axon contacts observed from altered expression of single cell surface proteins arise from a disturbance in the *balance* of factors expressed by each cell (Fambrough and Goodman, 1996; Winberg *et al.*, 1998a). For example, it may be that *lola* coordinately increases both general factors that promote overall neuromuscular interaction together with factors that specifically oppose inappropriate interactions.

Testing this model for *lola* function will require that we identify direct transcriptional targets of *lola.*

The phenotypes we observe from alteration of *lola* activity may suggest possible candidates for *lola* targets. For example, reduced SNb innervation can be produced by loss of *beat* expression in neurons, as discussed above, or by overexpression of the repulsive protein Semaphorin 2 in muscles (Matthes *et al.,* 1995). Conversely, increased interaction of SNb axons with their target muscles could reflect overproduction of proteins like Fasciclin 3 and Capricious that promote neuromuscular interaction, or diminished expression of proteins like Toll that limit neuromuscular interaction (Rose *et al.,* 1997). Indeed, we have observed that in *lola*$^{-}$ embryos, Fasciclin 3 immunoreactivity consistently seems less robust than is the case for their nonmutant siblings (EG, unpublished observations), perhaps consistent with upregulation of Fasciclin 3 by *lola.* Additional experiments will be required, however, to verify and quantify this apparent effect. Conversely, one protein whose expression is presumably *not* strongly affected by *lola* is Fasciclin 2: this is the marker we have used to visualize axon structure in these studies, and we have not observed obvious differences in Fasciclin 2 levels as we have altered *lola* activity. Before we can make strong predictions about which SNb regulatory proteins are potential *lola* targets, however, it will be necessary to determine whether LOLA activates or represses transcription in these neurons and muscles. Perhaps we can exploit the identification of *copia* as a direct *lola* target (Cavarec *et al.,* 1997) to assay whether LOLA is a positive or negative regulator of transcription in each of these cells *in vivo.* We also note that the direct targets of *lola* need not be the cell surface proteins themselves; it is equally possible that *lola* is part of a cascade of regulatory proteins.

Since *lola* is required for SNb-VLM interaction, and overexpression of *lola 4.7* in muscles further increases the interaction of these cells, it is striking that overexpression of *lola 4.7* in neurons should reduce their interaction. There are several possible explanations for this apparently paradoxical result. For example, activation in neurons of a growth cone-attracting factor may raise axon–axon adhesion excessively and thereby reduce the potential of growth cones to interact with muscles (Lin and Goodman, 1994). Alternatively, depending on the transcriptional cofactors recruited by LOLA in different cells, it may be that LOLA activates expression of adhesion proteins in muscles, but represses expression of proteins, perhaps even the same proteins, in neurons (Cavarec *et al.,* 1997). By either model, it may be that a key function of *lola* in neurons is to enhance neuromuscular specificity by limiting axon–target interaction (as discussed in the Introduction). Moreover, the existence of additional *lola* isoforms provides yet other possible mechanisms for the varied effects of overproduced *lola 4.7.* It is possible, for example, that some of the effect of overproducing *lola 4.7* in neurons arises from titration of endogenous *lola 3.8:* while overproduction of *lola 3.8* had no discernable dominant phenotype, this does not exclude the possibility that this isoform may have a positive function in SNb development in wild-type neurons (where it is most highly expressed). Finally, in the initial cloning of *lola* we reported the existence of a number of minor RNA species for which no corresponding cDNAs were successfully isolated (Giniger *et al.,* 1994). We cannot rule out the possibility that there exist as yet uncharacterized *lola* isoforms which, while present at relatively low abundance overall, nonetheless play a significant part in *lola* function in individual cells and that overproduction of *lola 4.7* might interfere with their function by formation of nonfunctional heterodimers or by titration of common cofactors. The extreme sensitivity of SNb development to the precise pattern and level of *lola* expression may also explain why we have been unable to rescue the *lola* mutant phenotype by GAL4-driven expression of single *lola* isoforms. Our results demonstrate that expression of *lola* even modestly above or below the wild-type level, in neurons, substratum cells, or target cells, leads to severe defects in SNb development. Thus, to test definitively which *lola* isoforms are actually required for SNb development, and in which tissues, it will be necessary either to isolate mutant alleles of *lola* which are specific for those isoforms and for expression in those tissues or else to identify fragments of the *lola* promoter which can reliably be used to drive expression of various *lola* minigenes in appropriate cells and at precisely wild-type levels.

In addition to its control of SNb morphogenesis, *lola* also regulates growth of CNS longitudinal axons between successive neuromeres, and growth of ISN axons along lateral peritracheal cells. It may be that some cell surface proteins act in multiple axon growth and guidance decisions, and that *lola* regulates some of the same genes in all three developmental contexts. We note, however, that *lola* is expressed very widely in the nervous system and in the mesoderm and further that *lola* includes a protein–protein interaction motif, the BTB domain, that is found in a large number of transcription factors, including many in *Drosophila* (Zollman *et al.,* 1994). For example, Abrupt is a BTB-containing protein that is expressed in the ventral muscles and is required for SNb morphogenesis, but is not required for other *lola*-dependent guidance decisions (Hu *et al.,* 1995). Perhaps LOLA forms different heteromeric complexes with distinct partners in different tissues and thereby can act in different ways to orchestrate growth cone function at different axonal choice points.

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